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14. ABSTRACT

Elevated serum levels of insulin-like growth factor 1 (IGF1) have been found in prostate cancer patients, and IGF1-related signal transduction is thought to be an important factor in the development of prostate cancers. The goals of this project are to discover small organic molecules that suppress IGF-activated prostate cancers by cell-based screening and to analyze their action mechanisms. During the funding period, we discovered, from our collection of synthetic compounds, the drug-like compound we call 125B11 that suppress IGF1-dependent growth of prostate cancer cells but not serum-dependent growth. We analyzed the mechanism of action of 125B11 to gain molecular insights into how IGF1 stimulates the growth of prostate cancer cells. DNA microarray and molecular biological experiments indicated that 125B11 blocks the activation of sterol regulatory element binding protein (SREBP), a transcription factor that activates specific genes involved in cholesterol synthesis, endocytosis of low density lipoproteins, and the synthesis of both saturated and unsaturated fatty acids. Our results suggest a novel crosstalk between fat/cholesterol metabolism and prostate cancer progression.

15. SUBJECT TERMS
Insulin-like growth factor, small molecule, chemical library, Chemical genetics, DNA microarray

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Introduction

Elevated serum levels of insulin-like growth factor 1 (IGF1) have been found in prostate cancer patients, and IGF1-related signal transduction is thought to be an important factor in the development of prostate cancers (*1*). The goals of this project are to discover small organic molecules that suppress IGF-activated prostate cancers by cell-based screening and to analyze their action mechanisms. We have been taking a unique two-step approach to discovering such molecules: we first examine the phenotypic effects of chemical library members (10,000 divergent drug-like compounds) on the insulin-induced adipogenic differentiation of cultured fibroblasts and, by using the adipogenesis profile, we then identify organic compounds that suppress IGF-mediated growth of prostate cancer cells. Our hypothesis is that some of the compounds that block the insulin-induced adipogenesis may suppress IGF-stimulated proliferation of malignant prostate cancers. The compounds may serve as a seed for a new type of anticancer drugs that could treat prostate cancers and also as a tool to understand the IGF signaling in malignant prostate cancers.

Body

Task 1:
To isolate small molecules that specifically inhibits the IGF-induced cell growth of prostate cancer cells

This task has been completed as described below.

Adipogenesis profiling for discovering anticancer agents (*2*). Our approach to discovering anticancer agents is based on the logic of genetics. In genetic screens, clear morphological phenotypes are often used just as a sensitive tool for discovering and analyzing genes whose primary functions are seemingly unrelated to the morphological phenotype. A good example is the use of eye morphology in the fruit fly *Drosophila melanogaster* as a genetic tool for the analysis of genes in disease-linked signaling pathways (*3*). Although human diseases associated with these pathways, such as cancer and neurodegenerative diseases, are seemingly unrelated to eye development, the use of eye morphology as a sensitive indicator enabled a systematic understanding of the disease-linked signaling events (*4-8*). We envisioned that clear morphological phenotypes of cells could similarly be used as a sensitive indicator of the drug effects that are not associated directly with the morphological phenotypes.

The morphological alteration we used is the differentiation of murine 3T3-L1 fibroblasts into adipocytes, one of the most drastic and sensitive morphological alterations in cultured mammalian cells (*9*). In the presence of insulin, 3T3-L1 cells undergo differentiation into adipocytes, which are visually distinct from the original cells because of the presence of oil droplets in the cytoplasm. The insulin-induced adipogenesis of 3T3-L1 cells involves a number

of disease-linked proteins such as PI3K, Ras, PPARγ, p38, or phosphodiesterases, and known drugs for a range of diseases have been reported to have phenotypic effects on the adipogenesis (*9-14*). A morphology-based adipogenesis screen of a chemical library could identify a pool of biologically active compounds with many distinct pharmacological effects including anticancer ones.

Adipogenesis profile of 10,000 divergent drug-like compounds (*2*). Our cell-morphology profiling of the 10,000-compound library identified 188 chemicals that clearly modulated the insulin-induced differentiation of 3T3-L1 cells at 20 ng/μL (**Fig. 1**): eighty-one compounds potentiated the adipogenesis, eighty-seven compounds completely blocked the differentiation, and thirteen compounds induced other morphological phenotypes such as adipocyte-like cells without oil droplets. The screen thus reduced pool of chemicals by 53 fold.

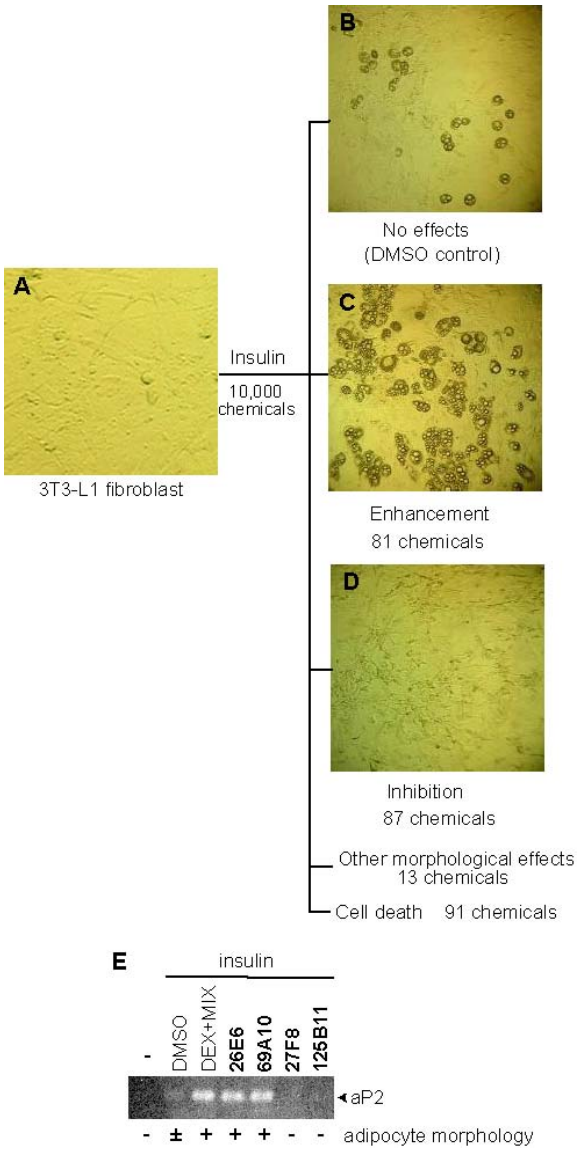


Fig. 1. Adipogenesis profiling of a library of 10,000 divergent drug-like compounds. 3T3-L1 cells have a morphology characteristic of fibroblasts (**A**). After chemical treatment in the presence of insulin, the cell morphology was examined under microscope. The control wells that are treated with 1% (v/v) DMSO have about 5% adipocytes (**B**). The compounds that enhanced adipogenesis more than five folds were scored to be adipogenesis-enhancing chemicals (**C**), and the compounds that completely inhibited adipogenesis without detectable cytotoxicity were scored to be adipogenesis-blocking chemicals (**D**). (**E**) RT-PCR analysis of adipocyte-specific aP2. 3T3-L1 cells were treated with

The adipogenesis-modulating activity of selected compounds were confirmed by RT-PCR analysis of aP2, an adipocyte-specific fatty acid-binding protein (an example is shown in **Fig. 1E**). The 188 adipogenesis-modulating chemicals that we found are apparently non-toxic for confluent 3T3-L1 cells and almost certainly modulate particular biologic responses in mammalian cells. In other words, the collection of 188 chemicals is a biology-focused chemical library.

Suppressors of IGF-activated prostate cancer cells (*2*). Both insulin and IGFs stimulate oncogenic signaling pathways including those of Ras-MAPK and PI3K-Akt, and overexpression of IGFs is often associated with cancer malignancy (*15*). Patients with IGF-overexpressing tumors tend to have severe hypoglycemia despite low levels of serum insulin (known as non-islet cell tumor hypoglycemia) (*16*), demonstrating a functional overlap between oncogenic IGFs and insulin in vivo. These considerations led to the hypothesis that the pool of the adipogenesis-blocking chemicals contains anticancer compounds that suppress the IGF-stimulated survival and proliferation of malignant tumor cells.

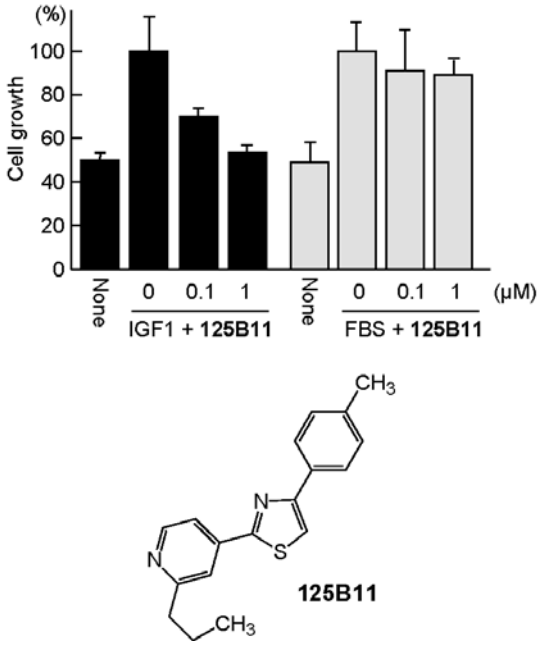


Fig. 2. Discovery of 125B11. 125B11 inhibited the IGF1-induced growth but not the serum-induced growth. DU-145 cells were treated with varied amounts of 125B11 in the presence of IGF1 or 2% fetal bovine serum (FBS).

For a chemical screen, we used DU-145 androgen-independent prostate cancer cells whose growth can be stimulated by IGF1 as much as by 2% serum. The pool of the 87 adipogenesis-blocking chemicals contained two analogous chemicals that specifically inhibited the IGF1-induced growth of DU-145 cells but had little effects on their serum-induced growth. One of them, 125B11, had the greatest differential activity; the simple drug-like thiazole derivative impaired the IGF1-induced growth at an IC_{50} of 0.1 μM but had little effects on the serum-dependent growth (**Fig. 2**). IGF1-induced phosphorylation of Akt and MAPK in DU-145 cells was unaffected by 125B11, suggesting that 125B11 inhibits the cell-proliferative function of IGF1 in a way independent of the known IGF1-signaling pathway.

Task 2:
To analyze mechanism of action of 125B11

This task has partially been completed, and the results suggested an interesting crosstalk between fat/cholesterol metabolism and prostate cancers.

Microarray experiments of 125B11

Gene expression profiling comparison in the drug-treated and -untreated cells by DNA microarray technology might reveal specific molecular pathways affected by the drugs. DU145 prostate cancer cells were treated with 125B11 or DMSO alone in the presence of IGF1, and extracted mRNA was analyzed by Affimetrix DNA micoarrays at the Microarray Facility in Baylor College of Medicine. To our surprise, we found that the genes downregulated by 125B11 included those known or likely to be controlled by sterol regulatory element binding protein (SREBP), a transcription factor that activates specific genes involved in cholesterol synthesis, endocytosis of low density lipoproteins, and the synthesis of both saturated and unsaturated fatty acids (*17*). These genes include LDL receptor and HMG-CoA reductase, a target of cholesterol-lowering drug statins. The microarray results were also verified by RT-PCR experiments. We hypothesized that 125B11, whether directly or indirectly, inhibits SREBP or its pathway.

Table 1

Microarray analysis of 125B11

Genes known to be controlled by SREBP

Genes relevant to sterol/fat synthesis

Downregulated by 125B11 in the presence of IGF1

P	D	0.707107	Hs.174140	NM_001096	ATP citrate lyase /FL=gb:NM_001096.1
P	D	0.707107	Hs.11223	NM_005896	isocitrate dehydrogenase 1 (NADP+), soluble /FL=gb:AF020038.1 gb:AF113917.1 gb:NM_005896.1 gb:AL136702.1
P	D	0.707107	Hs.268012	D89053.1	fatty-acid-Coenzyme A ligase, long-chain 3 /FL=gb:NM_004457.2 gb:D89053.1 gb:AF116690.1
P	D	0.707107	Hs.155976	AI650819	cullin 4B /FL=gb:NM_003588.1 gb:AB014595.1
P	D	0.707107	Hs.41693	BG252490	DnaJ (Hsp40) homolog, subfamily B, member 4 /FL=gb:U40992.2 gb:NM_007034.2
P	D	0.707107	Hs.14732	AL049699	malic enzyme 1, NADP(+)-dependent, cytosolic /FL=gb:NM_002395.2
P	D	0.707107	Hs.213288	S70123.1	low density lipoprotein receptor (familial hypercholesterolemia)
P	D	0.707107	Hs.6986	AL565516	Human glucose transporter pseudogene
P	D	0.659754	Hs.75616	NM_014762	seladin-1 /FL=gb:AF261758.1 gb:BC004375.1 gb:NM_014762.1
P	D	0.659754	Hs.77393	NM_002005	farnesyl diphosphate synthase (farnesyl pyrophosphate synthetase, dimethylallyltranstransferase, geranyltranstransferase
P	D	0.659754	Hs.132898	NM_005063	fatty acid desaturase 1
P	D	0.659754	Hs.274398	BC002654	Homo sapiens, Similar to tubulin, beta, 4, clone MGC:4083, mRNA, complete cds
P	D	0.659754		BC005838	Homo sapiens, tubulin, beta 5
P	D	0.659754	Hs.115283	BF978872	dihydrolipoamide S-acetyltransferase (E2 component of pyruvate dehydrogerase complex)
P	D	0.659754	Hs.159154	AL565749	tubulin, beta, 4
P	D	0.659754	Hs.179817	AF167438	Homo sapiens androgen-regulated short-chain dehydrogenasereductase 1 (ARSDR1)
P	D	0.615572	Hs.213288	NM_000527	low density lipoprotein receptor (familial hypercholesterolemia) /FL=gb:NM_000527.2
P	D	0.615572	Hs.75105	NM_006579	emopamil-binding protein (sterol isomerase) /FL=gb:NM_006579.1
P	D	0.615572	Hs.74304	NM_002705	Homo sapiens periplakin (PPL)
A	D	0.615572	Hs.268515	NM_002431	meningioma (disrupted in balanced translocation) 1 /FL=gb:NM_002430.1
P	D	0.615572	UG=Hs.31	AF096304	Homo sapiens putative sterol reductase SR-1 (TM7SF2) transmembrane 7 superfamily member 2 /FL=gb:AF096304.1
P	D	0.615572	Hs.93199	D63807.1	lanosterol synthase (2,3-oxidosqualene-lanosterol cyclase) /FL=gb:D63807.1
P	D	0.574349	Hs.119597	AB032261	stearoyl-CoA desaturase (delta-9-desaturase) /FL=gb:AF097514.1 gb:NM_005063.1 gb:AB032261.1
P	D	0.574349	Hs.174140	AI971281	ATP citrate lyase /FL=gb:NM_001096.1
P	D	0.574349	Hs.171825	NM_003670	basic helix-loop-helix domain containing, class B, 2 /FL=gb:AB004066.1 gb:NM_003670.1
P	D	0.574349	Hs.79103	AW235052	cytochrome b5 outer mitochondrial membrane precursor /FL=gb:BC004373.1 gb:NM_030579.1
P	D	0.574349	Hs.11806	NM_001360	dehydrocholesterol reductase /FL=gb:BC000054.1 gb:AF034544.1 gb:AF067127.1 gb:AF096305.1 gb:NM_001360.1
P	D	0.574349	Hs.3838	NM_006625	serum-inducible kinase /FL=gb:AF059617.1 gb:NM_006622.1 gb:AF223574.1
P	D	0.574349	Hs.213288	AI861942	low density lipoprotein receptor (familial hypercholesterolemia) /FL=gb:NM_000527.2
P	D	0.574349	Hs.93199	AW084501	lanosterol synthase (2,3-oxidosqualene-lanosterol cyclase) /FL=gb:NM_002340.1 gb:U22526.1
P	D	0.574349	Hs.5920	NM_005475	UDP-N-acetylglucosamine-2-epimeraseN-acetylmannosamine kinase
P	D	0.574349	Hs.1524	NM_003810	tumor necrosis factor (ligand) superfamily, member 9 /FL=gb:NM_003811.1 gb:U03398.1
P	D	0.574349	Hs.278544	BC000408	acetyl-Coenzyme A acetyltransferase 2 (acetoacetyl Coenzyme A thiolase) /FL=gb:BC000408.1
P	D	0.574349	Hs.174140	U18197.1	ATP citrate lyase /FL=gb:U18197.1
P	D	0.574349	Hs.7232	BE855983	acetyl-Coenzyme A carboxylase alpha /FL=gb:NM_000664.1 gb:U19822.1
P	D	0.535887	Hs.226213	NM_000781	cytochrome P450, 51 (lanosterol 14-alpha-demethylase) /FL=gb:U23942.1 gb:NM_000786.1 gb:D55653.1
A	D	0.535887	Hs.268490	NM_004759	nuclear receptor subfamily 0, group B, member 1 /FL=gb:NM_000475.2
P	D	0.535887	Hs.48876	AA872727	farnesyl-diphosphate farnesyltransferase 1 /FL=gb:L06070.1 gb:L06105.1 gb:NM_004462.1
P	D	0.535887	Hs.65370	NM_006330	lipase, endothelial /FL=gb:AF118767.1 gb:NM_006033.1
P	D	0.535887	Hs.14779	AK000162	acetyl-CoA synthetase
P	D	0.535887	Hs.44499	U59479.1	pinin, desmosome associated protein /DEF=Human neutrophil protein mRNA, partial cds.
P	D	0.5	Hs.154654	AU144855	cytochrome P450, subfamily I (dioxin-inducible), polypeptide 1 (glaucoma 3, primary infantile)
P	D	0.5	Hs.11899	AL518627	3-hydroxy-3-methylglutaryl-Coenzyme A reductase /FL=gb:M11058.1 gb:NM_000859.1
P	D	0.5	Hs.2178	NM_003526	H2B histone family, member Q /FL=gb:NM_003528.1
P	D	0.5	Hs.130607	NM_000431	mevalonate kinase (mevalonic aciduria) /FL=gb:M88468.1 gb:NM_000431.1
P	D	0.5	Hs.284244	NM_002006	fibroblast growth factor 2 (basic) /FL=gb:M27968.1 gb:NM_002006.1
P	D	0.5	Hs.71465	AF098865	squalene epoxidase /FL=gb:D78130.1 gb:AF098865.1 gb:NM_003129.2
P	D	0.5	Hs.288031	D85181.1	sterol-C5-desaturase (fungal ERG3, delta-5-desaturase)-like /FL=gb:D85181.1
A	D	0.5		L32662.1	prostaglandin E2 receptor /DB_XREF=gi:484163 /FL=gb:L32662.1
P	D	0.5	Hs.81412	D80010.1	lipin 1
P	D	0.466517	Hs.11899	NM_000859	3-hydroxy-3-methylglutaryl-Coenzyme A reductase /FL=gb:M11058.1 gb:NM_000859.1
P	D	0.466517	Hs.79440	NM_006547	IGF-II mRNA-binding protein 3 /FL=gb:U97188.1 gb:U76705.1 gb:AF117108.1 gb:NM_006547.1
P	D	0.466517	Hs.75318	AL565074	tubulin, alpha 1 (testis specific)
P	D	0.466517	Hs.226213	U40053	cytochrome P450, 51 (lanosterol 14-alpha-demethylase)
P	D	0.466517	Hs.187573	NM_016371	hydroxysteroid (17-beta) dehydrogenase 7 /FL=gb:AF098786.2 gb:NM_016371.1
P	D	0.435275	Hs.76038	BC005247	isopentenyl-diphosphate delta isomerase /FL=gb:BC005247.1
P	D	0.435275	Hs.57698	BC000245	NAD(P) dependent steroid dehydrogenase-like; H105e3 /FL=gb:BC000245.1 gb:U47105.2 gb:NM_015922.1
P	D	0.353553	Hs.77910	NM_002130	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 (soluble) /FL=gb:BC000297.1 gb:L25798.1 gb:NM_002130.1
P	D	0.329877	Hs.56205	BE300521	insulin induced gene 1 /FL=gb:NM_005542.1
P	D	0.329877	Hs.154654	NM_000104	cytochrome P450, subfamily I (dioxin-inducible), polypeptide 1 (glaucoma 3, primary infantile) /FL=gb:NM_000104.2 gb:U0
P	D	0.329877	Hs.3828	AI189359	mevalonate (diphospho) decarboxylase /FL=gb:U49260.1 gb:BC000111.1 gb:NM_002461.1

125B11 blocks cleavage of SREBP in cells

Goldstein, Brown, and colleagues have demonstrated that in the presence of sterol, 125 kDa SREBP is located endoplasmic reticulum membranes. When cellular sterol concentrations were lowered, the amino terminal 68 kDa domain of SREBP is released from the membrane, entered the nucleus, and bound to SREs in the promoters of SREBP-target genes. In contrast, the cleavage of SREBPs was prevented when cells were loaded with cholesterol/oxysterols; the result was low nuclear levels of SREBP and low rates of transcription of SREBP target genes (**Fig. 3**) (***18***).

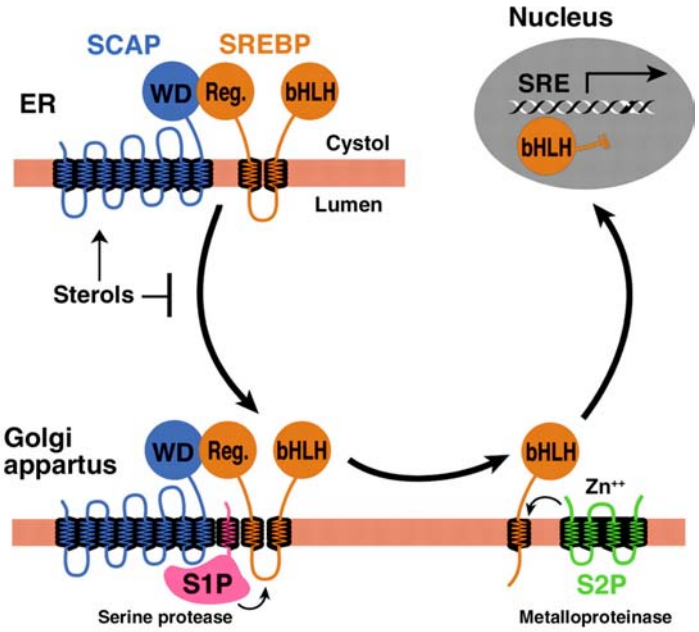


Fig. 3 Regulation of SREBP by sterols.

We first examined if 125B11 inhibits the transcription of a reporter gene under control of five tandem repeats of a typical SREBP binding site. The SREBP-responsive reporter gene was constitutively active in DU145 prostate cancer cells, and addition of 125B11 into the culture inhibited the expression of the reporter gene in a dose-dependent manner, whereas the control reporter with the SV40 promoter had no detectable effects.

We next examined if the cleavage of SREBP is blocked by 125B11 in cells. Western blot analysis showed that treatment with 5 μM of 125B11 increased the amounts of uncleaved 125Kda SREBP (**Fig. 4**).

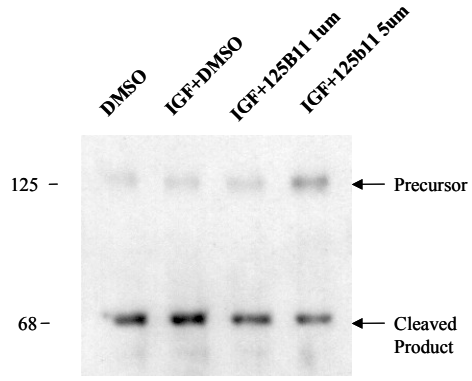


Fig. 4 Effect of 125B11 on SREBP-1 protein in DU145 cells. DU145 cells were seeded onto 6-well plates at a density of 2.5x10^5 with serum-free media for 16h before receive chemicals. Cells were then incubated for 6h with DMSO (line 1) or 1ug/ml IGF (lane 2) or IGF with 1uM 125B11 (lane 3) or IGF with 5uM 125B11 (lane 4) in non-serum medium. The levels of SREBP-1 in cell lysates were examined by Western blotting with an anti-SREBP-1 antibody.

Since 125B11 blocks the cleavege of SREBP, 125B11 should prevent SREBP from entering the nucleus. To confirm that, we examined

the effect of 125B11 on the nuclear translocation of SREBP by immunostaining. As shown in **Fig. 5**, it is evident that the amounts of nuclear SREBP1 is decreased.

It remains unknown how 125B11 inhibits the cleavage of SREBP. In sterol-deprived cells, a protein called SCAP functions as a chaperone protein and transports the 125 kDa SREBPs from the endoplasmic reticulum to the Golgi where two proteases (S1P and S2P) cleave SREBP to generate 68Kda SREBP (**Fig. 3**) (*17*). In contrast, in cholesterol-loaded cells, SCAP and SREBP fail to migrate to the Golgi and the 125 kDa SREBP remains in ER. Thus, under these conditions, maturation and nuclear localization of SREBPs are reduced. 125B11 may modulate one of these transport/cleavage processes.

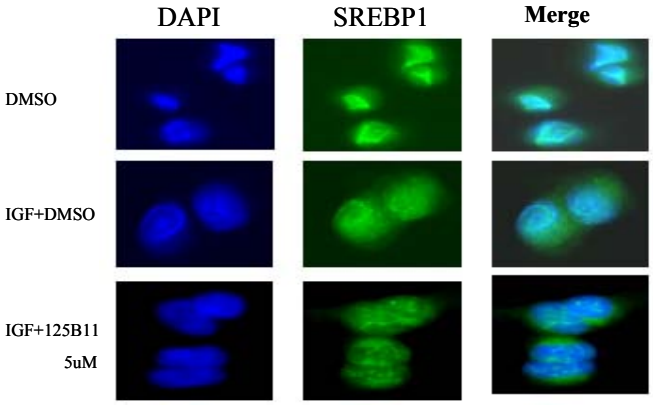


Fig. 5. 125B11 blocks the nuclear localization of SREBP1. DU145 cells were seeded on a cover slide in serum free MEM. After one day, the cells were treated with 125B11 or DMSO alone in the presence of IGF. Six hours after the treatment, the cells were permeabilized and stained with an antibody against SREBP1.

SREBP plays a role in IGF1-dependent growth of prostate cancers.

Our results suggest that 125B11 suppress IGF1-dependent growth of prostate cancer cells by blocking SREBP activation. If this hypothesis is correct, then siRNA knockdown of SREBP suppress IGF1-dependent growth of prostate cancers. We stably transfected siRNA expression vector of SREBP to DU145 cells and examined its growth in the presence of IGF1. As shown in **Fig. 6**, the knowckdown cells seem to be irresponsive to IGF1. This result suggest that SREBP plays an essential role in IGF1-dependent growth and prostate cancer progression. Our continued experiments may reveal an interesting crosstalk between cholesterol metabolism and prostate cancers.

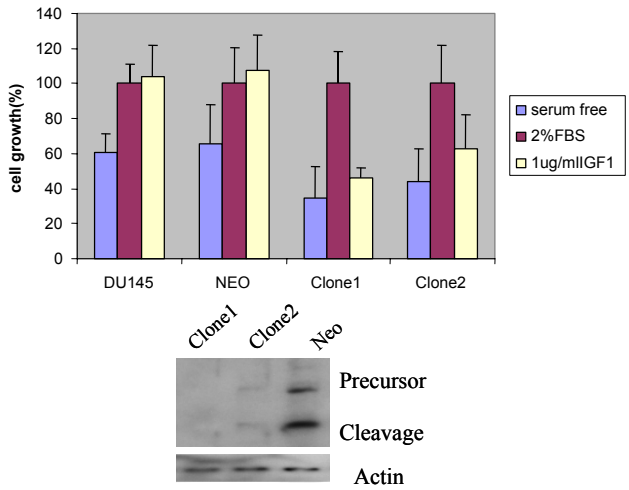


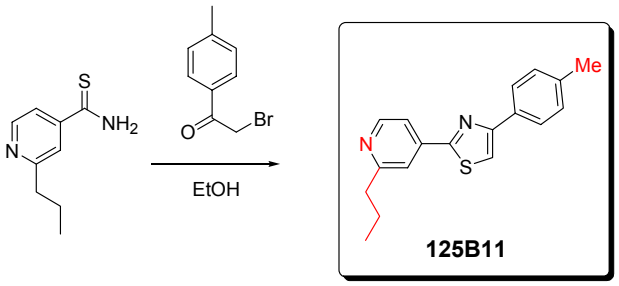
Fig. 6. siRNA knockdown of SREBP-1 blocks IGF-dependent growth of DU145 prostate cancer cells. The extents of SREBP1 knockdown in clones 1 and 2 are shown in the lower panel.

Synthesis of 125B11 analogs

The key reaction of the synthesis of 125B11 is shown in Scheme 1. This scheme enabled gram-scale preparation of 125B11 and quick preparation of 125B11. We have synthesized ten 125B11 analogs with the

variation of the methyl and propyl positions indicated in red. The analogs were assayed for their ability to block the cleavage of SREBP1. Unfortunately, we failed to find a 125B11 analog better than 125B11 in the ten molecules. We continue to synthesize more analogs with multiple synthetic schemes to obtain a complete set of structure-activity relationship. Such a study would identify the position suited for biotinylation for isolating molecular target of 125B11.

Scheme 1



125B11 represents the first small molecule inhibitor of SREBP.

SREBP is the master regulator of fat and cholesterol synthesis (In fact, knockdown of SREBP1 in 3T3-L1 cells completely blocked the insulin induced adipogenesis in our hands). As far as we notice, no small molecule inhibitors of SREBP have been discovered. Although we found this unique molecule from the study of the IGF-dependent growth of prostate cancer cells, 125B11 may serve as a tool to investigate how cells regulate the fat/cholesterol synthesis.

We collaborated with Dr. Salih J. Wakil in our department, a leading scientist in the field of fatty acid synthesis, to examine the effects of 125B11 on metabolism in mice. The results are summarized in **Fig. 7**. Two week treatment with 125B11 apparently reduced body weight in mice while the treatment had no detectable effects on food intake. Livers in mice were excised and their extracts were analyzed by western blots. The results showed that 125B11 blocks the cleavage of SREBP1 and reduced the expression of fatty acid synthase in liver.

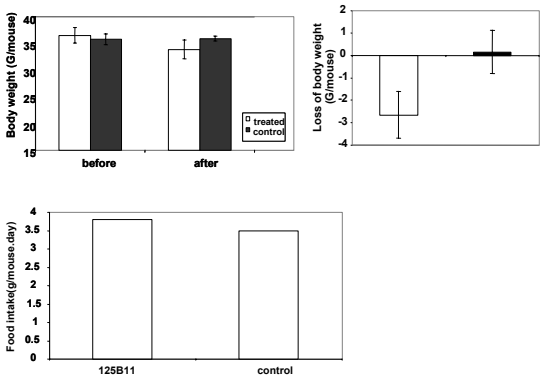


Fig. 7. Two week treatment with 125B11 apparently reduced body weight in mice while the treatment had no detectable effects on food

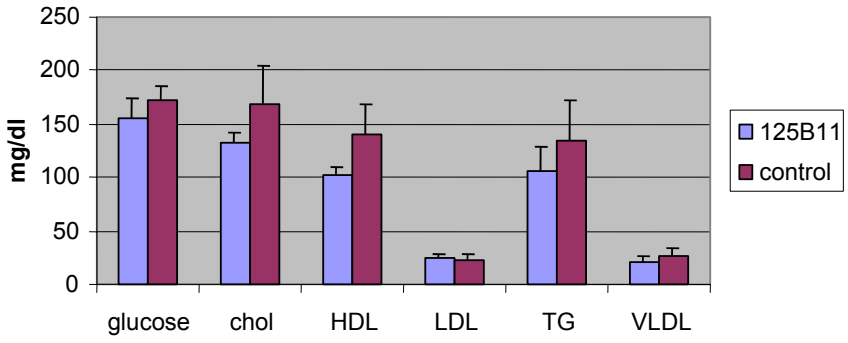


Fig. 8. Blood constituents of the mice treated with 125B11.

Blood samples from the mice were also analyzed. As shown in **Fig. 8**, levels of cholesterol and triglyceride were lowered by the treatment. These results in mice support the notion that 125B11 is an inhibitor of SREBP.

Key research accomplishments

- Obtained experimental results supporting our hypothesis that adipogenesis profiling of organic molecules is useful for discovering IGF suppressing compounds
- Discovered the unique compound 125B11
- Obtained evidence suggesting that 125B11 has an unprecedented mechanism of action.
- Identified SREBP-responsive genes as genes downregulated by 125B11
- Obtained evidence suggesting that 125B11 blocks the cleavage of SREBP
- Obtained results suggesting that SREBP plays an essential role in IGF1-dependent growth of prostate cancers.
- Synthesized 125B11 analogs
- Conducted animal experiments showing that 125B11 blocks SREBP in mice.

Reportable outcomes

- Identification of bioactive molecules by adipogenesis profiling of organic compounds
  Choi, Y., Kawazoe, Y., Murakami, K., Misawa, H., **Uesugi, M.**
  J. Biol. Chem. 278, 7320-7324 (2003).
- Identification of bioactive molecules by adipogenesis profiling of organic compounds
  Choi, Y., Kawazoe, Y., Murakami, K., **Uesugi, M.**
  FASEB Journal, 17 (4): A605-A605 (2003).
- Results were discussed in 2004 Prostate Cancer Foundation Retreat

Conclusion

Fat cell differentiation *per se* has no direct link to suppression of IGF1-activated prostate cancer cells. Nevertheless, our proof-of-principle study using a 10,000-compound library successfully identified a non-cytotoxic IGF1-suppressing compound, 125B11. Our analysis of 125B11 indicated that 125B11 selectively inhibit the activation of SREBP transcription factor, indicating the role of SREBP in IGF1-dependent growth of prostate cancers. The results suggest a new link between fat/cholesterol metabolism and prostate cancer progression. We continue to understand how 125B11 blocks the cleavage of SREBP. Such studies may lead to the discovery of a new drug target for prostate cancers or metabolic diseases.

References

1. B. Djavan, M. Waldert, C. Seitz, M. Marberger, *World J Urol* **19**, 225-33 (Aug, 2001).
2. Y. Choi, Y. Kawazoe, K. Murakami, H. Misawa, M. Uesugi, *J Biol Chem* **278**, 7320-4 (Feb 28, 2003).
3. B. J. Thomas, D. A. Wassarman, *Trends Genet* **15**, 184-90 (May, 1999).
4. D. A. Wassarman, M. Therrien, G. M. Rubin, *Curr Opin Genet Dev* **5**, 44-50 (Feb, 1995).
5. H. Luo, C. R. Dearolf, *Bioessays* **23**, 1138-47 (Dec, 2001).
6. K. McCall, H. Steller, *Trends Genet* **13**, 222-6 (Jun, 1997).
7. R. Burke, K. Basler, *Curr Opin Neurobiol* **7**, 55-61 (Feb, 1997).
8. K. T. Min, S. Benzer, *Science* **284**, 1985-8 (Jun 18, 1999).
9. E. D. Rosen, B. M. Spiegelman, *Annu Rev Cell Dev Biol* **16**, 145-71 (2000).
10. D. J. Klemm *et al.*, *J Biol Chem* **276**, 28430-5 (Jul 27, 2001).
11. I. C. Ho, J. H. Kim, J. W. Rooney, B. M. Spiegelman, L. H. Glimcher, *Proc Natl Acad Sci U S A* **95**, 15537-41 (Dec 22, 1998).
12. P. Dowell, C. Flexner, P. O. Kwiterovich, M. D. Lane, *J Biol Chem* **275**, 41325-32 (Dec 29, 2000).
13. J. A. Engelman, M. P. Lisanti, P. E. Scherer, *J Biol Chem* **273**, 32111-20 (Nov 27, 1998).
14. J. A. Engelman *et al.*, *J Biol Chem* **274**, 35630-8 (Dec 10, 1999).
15. H. Yu, T. Rohan, *J Natl Cancer Inst* **92**, 1472-89 (Sep 20, 2000).
16. W. H. Daughaday, *Diabetes Rev* **3**, 62-72 (1995).
17. P. A. Edwards, D. Tabor, H. R. Kast, A. Venkateswaran, *Biochim Biophys Acta* **1529**, 103-13 (Dec 15, 2000).
18. M. S. Brown, J. L. Goldstein, *Cell* **89**, 331-40 (May 2, 1997).

Appendix

- One reprint copy of the paper that we published in *J. Biol. Chem*.

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Identification of Bioactive Molecules by Adipogenesis Profiling of Organic Compounds*[S]

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An important step in the postgenomic drug discovery is the construction of high quality chemical libraries that generate bioactive molecules at high rates. Here we report a cell-based approach to composing a focused library of biologically active compounds. A collection of bioactive non-cytotoxic chemicals was identified from a divergent library through the effects on the insulin-induced adipogenesis of 3T3-L1 cells, one of the most drastic and sensitive morphological alterations in cultured mammalian cells. The resulting focused library amply contained unique compounds with a broad range of pharmacological effects, including glucose-uptake enhancement, cytokine inhibition, osteogenesis stimulation, and selective suppression of cancer cells. Adipogenesis profiling of organic compounds generates a focused chemical library for multiple biological effects that are seemingly unrelated to adipogenesis, just as genetic screens with the morphology of fly eyes identify oncogenes and neurodegenerative genes.

A complete analysis of human genome is anticipated to produce an unprecedented number of potential drug targets. The development of high throughput assays for these genomic pseudotargets may be a challenging but important step for not limiting drug discovery to the "relatively easy" targets such as G-protein-coupled receptors or particular enzymes. An alternative or complementary effort is the construction of high quality chemical libraries that generate bioactive molecules at higher rates. The small size of the focused libraries would lower the cost of screening processes and enable unique low throughput screens, extending the scope of assays for the genomic targets and for a given therapeutic effect.

Our approach to constructing a focused chemical library is based on the logic of genetics. In genetic screens, clear morphological phenotypes are often used just as a sensitive tool for discovering and analyzing genes whose primary functions are seemingly unrelated to the morphological phenotype. A good example is the use of eye morphology in the fruit fly *Drosophila melanogaster* as a genetic tool for the analysis of genes in disease-linked signaling pathways (1). Although human diseases associated with these pathways, such as cancer and neurodegenerative diseases, are seemingly unrelated to eye development, the use of eye morphology as a sensitive indicator enabled a systematic understanding of the disease-linked signaling events (2–6). We envisioned that clear morphological phenotypes of cells could similarly be used as a sensitive indicator of the drug effects that are not associated directly with the morphological phenotypes.

The morphological alteration we used is the differentiation of murine 3T3-L1 fibroblasts into adipocytes, one of the most drastic and sensitive morphological alterations in cultured mammalian cells (7). In the presence of insulin, 3T3-L1 cells undergo differentiation into adipocytes, which are visually distinct from the original cells because of the presence of oil droplets in the cytoplasm (Fig. 1). The insulin-induced adipogenesis of 3T3-L1 cells involves a number of disease-linked proteins such as phosphatidylinositol 3-kinase, Ras, peroxisome proliferator-activated receptor γ, p38, or phosphodiesterases, and known drugs for a range of diseases have been reported to have phenotypic effects on the adipogenesis (7–12). A morphology-based adipogenesis screen of a chemical library could identify a pool of biologically active compounds with many distinct pharmacological effects. Here we report a proof-of-principle study using a library of 10,000 divergent compounds.

EXPERIMENTAL PROCEDURES

Adipogenesis Profiling—3T3-L1 fibroblasts were plated in 96-well plates at a density of 5×10^4 cells/well and allowed to reach maximal confluence. The confluent cells were treated individually with 20 ng/μl of a chemical for 3 days in 100 μl of Dulbecco's modified Eagle's medium containing of insulin (5 μg/ml) and 10% fetal bovine serum (FBS).[1] After the removal of insulin and the chemical, the cells were further maintained typically for 8 days with the replacement of media every 3 days. The effects of chemicals on the adipogenesis were evaluated under microscope. The control wells with 1% (v/v) Me_2SO had ~5% adipocytes. The compounds that enhanced the adipogenesis >5-folds were scored to be adipogenesis-enhancing chemicals, and the ones that completely inhibited adipogenesis without detectable toxicity were scored to be adipogenesis-blocking chemicals. The effects of these chemicals were confirmed multiple times by multiple laboratory members. Cell viability was monitored by trypan blue exclusion and by counting cell numbers.

Reverse Transcription (RT)-PCR—Total RNA was isolated with TRI-reagent (Molecular Research Center) at day 7 (aP2) or day 3 (osteocalcin). 5 μg of total RNA was reverse-transcribed to cDNA by using oligo(dT) primer with avian myeloblastosis virus reverse transcriptase for 60 min at 42 °C. The cDNA was then amplified by using Ex*Taq*

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[S] The on-line version of this article (available at http://www.jbc.org) contains Figs. 1 and 2.

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[1] The abbreviations used are: FBS, fetal bovine serum; RT, reverse transcription; IL, interleukin; TNF, tumor necrosis factor; ELISA, enzyme-linked immunosorbent assay; IGF, insulin-like growth factor; SEAP, secreted alkaline phosphatase; AP, activating protein; MAPK, mitogen-activated protein kinase; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide.

(Takara) with following primer pairs: 5′-AACACCGAGATTTCCTTCAA-3′ and 5′-TCACGCCTTTCATAACACAT-3′ for aP2; 5′-TCTGACAAACCTTCATGTCC-3′ and 5′-AAATAGTGATACCGTAGATGCG-3′ for osteocalcin. The amplification conditions are as follows: 95 °C (30 s), 60 °C (30 s), 72 °C (30 s) for 23 cycles (aP2) or 30 cycles (osteocalcin).

Glucose Uptake Study—3T3-L1 fibroblasts were induced to differentiate into adipocytes by incubation in a medium containing 10% FBS, 1 μM dexamethasone, 0.5 mM methylisobutylxanthine, and 1.7 μM insulin. After 2 days, the medium was switched to the one containing 10% FBS and 1.7 μM insulin for 2 days and then to a normal 10% FBS medium for 3 days. After the total of 7 days, almost 100% 3T3-L1 cells were differentiated into adipocytes. These fully differentiated cells were treated on 24-well plates with varied concentrations of chemicals (0.1% Me_2SO) for 24 h and then incubated with 100 nM insulin and 2-[^{3}H]deoxyglucose. The cells were extensively washed, and their radioactivity was measured by scintillation counting. All of the samples were tested in duplicate.

Cytokine Production Assay—For the analysis of IL-6 and TNF-α, the mouse macrophage cell line RAW264.7 was used. Cells were seeded onto 96-well plates, and the cytokine production was induced by adding 10 μg/ml lipopolysaccharide. Upon stimulation, each one of the adipogenesis-enhancing chemicals was also added to the culture at varied concentrations. After incubating for 48 h at 37 °C, the cytokine concentrations in the culture supernatants were measured by ELISA. For the analysis of IL-2, the mouse thymoma cell line EL-4 was used, and the IL-2 production was induced by adding phorbol ester and ionophore. The effects of chemicals on the IL-2 production were similarly examined by ELISA. All of the samples were tested in triplicate.

Mineralization Assay—The clonal osteoblastic cell line MC3T3-E1, clone 14, was grown in α-minimum Eagle's medium supplemented with 10% FBS until confluent in 96-well plates. For induction of mineralization, the cells were further incubated with 50 μg/ml ascorbic acid and 10 mM β-glycerophosphate in the presence or absence of chemicals. On day 14, the cells were washed with phosphate-buffered saline, fixed in 10% formalin, and washed with distilled water. Bonelike mineral formation was evaluated by examining the area stained by 2% (w/v) Alizarin Red S (pH 4.2).

Assays for Insulin-like Growth Factor (IGF)-activated Cancer Cells—The adipogenesis-blocking chemicals were assayed for their ability to inhibit the growth of IGF-activated cancer cells. For the discovery of inhibitors of IGF2, we used five distinct human hepatocellular carcinoma cell lines, Hep-G2, SK-Hep-1, and three lines that we recently characterized.[2] Three of them produce IGF2 at high levels, whereas two express ~10 times less amounts of IGF2 as measured by ELISA, RT-PCR, and DNA microarray experiments. Treatment with a neutralizing antibody against IGF2 selectively inhibited the growth of the IGF2-overexpressing cell lines but had little effects on that of the cell lines with low levels of IGF2. Thus, these cell lines served as an excellent system for discovering the chemicals that selectively impair the growth of IGF2-overexpressing hepatocellular carcinoma cells. For cell viability assays, IGF2-expressing cells were plated at a density of 4×10^3 onto 96-well plates. After a 24-h incubation, the cells were treated with varied amounts of chemicals for 72 h. The effects of chemicals were evaluated by microscopic observation and MTT assay. All of the samples were tested at least three times. For reporter gene assays, IGF2-expressing cells were transfected with a reporter construct in which a gene encoding secreted alkaline phosphatase (SEAP) is controlled by the IGF2 promoter, AP-1 sites, NFkB sites, or the SV40 promoter. After 24 h, the transfected cells were treated with 94G6 (0.1 μM) for 8 h. SEAP activity was measured through fluorescence change of methylumbelliferyl phosphate. The experiments were repeated six times. For the discovery of inhibitors of IGF1, we used DU-145, a human androgen-independent prostate cancer cell whose growth can be stimulated by IGF1 in a non-serum medium (13). Chemicals that inhibit the IGF1-induced growth of DU-145 but not its serum-dependent growth were searched in the focused library of adipogenesis-blocking chemicals. DU-145 cells were seeded onto 96-well plates at a density of 2,000 cells/well in the presence of 1 μg/ml IGF1 or 2% FBS. After 24 h, chemicals were added to the culture at varied concentrations. Cell proliferation was estimated by MTT assays after 3 days. The experiments were performed in triplicate.

RESULTS

Adipogenesis Profiling of 10,000 Divergent Compounds—The divergent chemical library used for our case study was a Prime Collection 2000 Format Q (ChemBridge). In this format, 10,000 druglike molecules are rationally preselected to form a library that covers the maximum pharmacore diversity with the minimum number of compounds. Two academic groups (14, 15) have reported successful isolations of unique compounds from a similar chemical library, indicating that this type of chemical library contains a diverse set of compounds that are suited for a proof-of-principle study. Our cell morphology profiling of the 10,000-compound library identified 188 chemicals that clearly modulated the insulin-induced differentiation of 3T3-L1 cells at 20 ng/μl (Fig. 1): 81 compounds potentiated the adipogenesis; 87 compounds completely blocked the differentiation; and

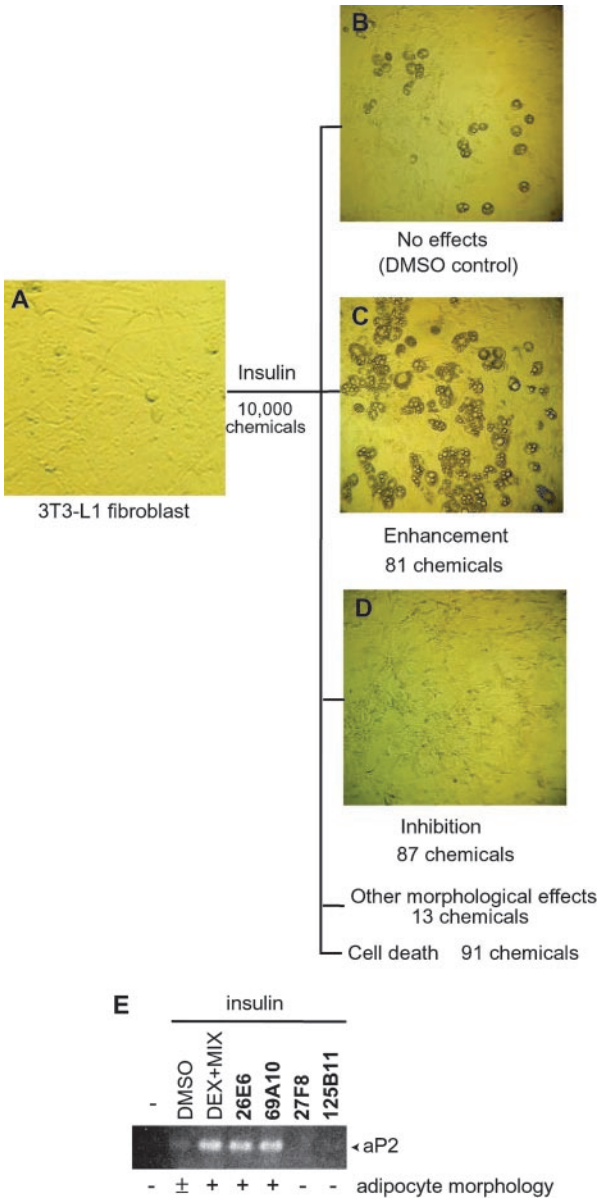

FIG. 1. **Adipogenesis profiling of a library of 10,000 divergent druglike compounds.** *A*, 3T3-L1 cells have a morphology characteristic of fibroblasts. After chemical treatment in the presence of insulin, the cell morphology was examined under microscope. *B*, the control wells that are treated with 1% (v/v) Me_2SO (*DMSO*) have ~5% adipocytes. The compounds that enhanced adipogenesis >5-folds were scored to be adipogenesis-enhancing chemicals (*C*), and the compounds that completely inhibited adipogenesis without detectable cytotoxicity were scored to be adipogenesis-blocking chemicals (*D*). *E*, RT-PCR analysis of adipocyte-specific aP2. 3T3-L1 cells were treated with chemicals for 3 days, and total RNA was isolated at day 7. Typical results of four representative compounds are shown along with the positive control of 1 μM dexamethasone (*DEX*) and 0.5 mM methylisobutylxanthine (*MIX*).

[2] K. Murakami and M. Uesugi, unpublished data.

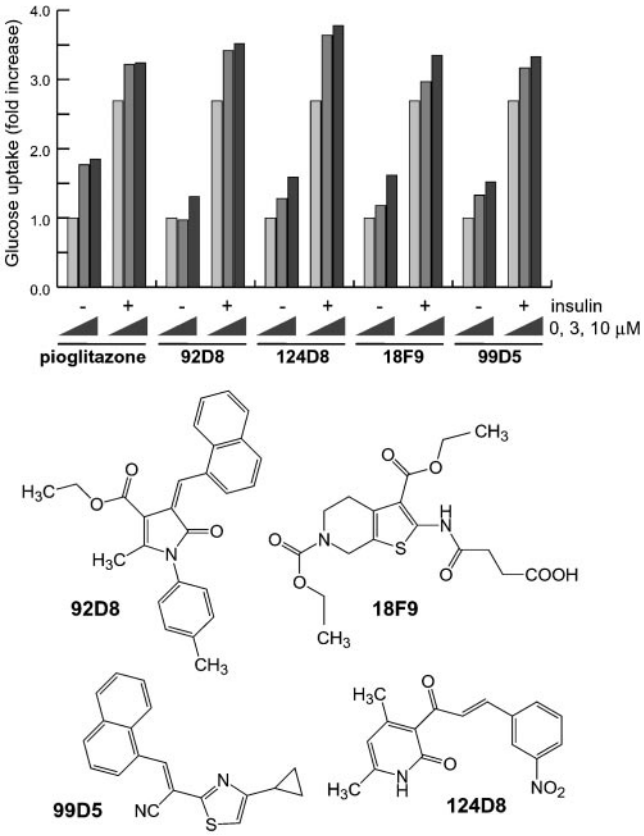

FIG. 2. **Identification of glucose-uptake enhancers.** Fully differentiated adipocytes were treated with 3 or 10 μM chemicals and 2-[^{3}H]deoxyglucose in the presence or absence of 100 nM insulin on 24-well plates. Glucose uptake was measured by scintillation counting. The results of the best four chemicals are shown.

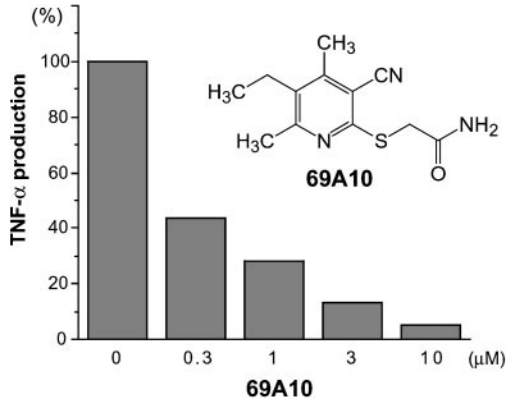

FIG. 3. **Inhibition of TNF-α production by 69A10.** Macrophage RAW264.7 cells were seeded onto 96-well plates, and TNF-α was induced by adding lipopolysaccharide. Upon the stimulation, 69A10 was added to the culture. After incubating for 48 h, the TNF-α concentrations were measured by ELISA.

13 compounds induced other morphological phenotypes such as adipocyte-like cells without oil droplets. Thus, the screen reduced a pool of chemicals by 53-fold. The adipogenesis-modulating activity of selected compounds was confirmed by RT-PCR analysis of aP2, an adipocyte-specific fatty acid-binding protein (an example is shown in Fig. 1*E*). The 188 adipogenesis-modulating chemicals that we found are apparently non-toxic for confluent 3T3-L1 cells and almost certainly modulate particular biologic responses in mammalian cells. The chemical structures of adipogenesis-enhancing and -blocking compounds are disclosed in supplementary Figs. 1 and 2.

Glucose-uptaking Insulin Sensitizers—We first focused on the 81 chemicals that potentiated the insulin-induced adipogenesis. Their insulin-sensitizing activity in the adipocyte differentiation suggests that some of them enhance the insulin-induced glucose uptake with anti-diabetic properties. This prediction was supported by the fact that the thiazolidinedione family of anti-diabetic drugs enhances the adipogenesis of 3T3-L1 cells through the activation of peroxisome proliferator-activated receptor γ, a nuclear receptor that plays an important role in adipocyte differentiation (16). In fact, among the adipogenesis-enhancing compounds, nine had a structural element chemically equivalent to thiazolidinedione. These known chemicals were eliminated, and the remaining 72 chemicals were assayed for their ability to potentiate insulin-induced glucose uptake in cultured adipocytes. The 72 compounds contained as many as 11 molecules that enhanced the glucose uptake at comparable levels with that of pioglitazone, a clinically used anti-diabetic drug, demonstrating the validness of our approach. Four of them exhibited insulin-sensitizing activity stronger than pioglitazone at 10 μM, and the most potent one was 124D8 (Fig. 2). Its kinase-inhibitor-like structure is novel as an insulin sensitizer and appears to modulate the function of insulin independently from the major insulin pathways because 124D8 had no effects on the phosphorylation of Akt and MAPK in 3T3-L1 cells. Adipogenesis profiling of a larger chemical library is likely to generate a number of glucose-uptaking compounds with a novel mechanism of action.

Inhibitors of Inflammatory Cytokine Production—Recent studies (7, 12) suggest a cross-talk between insulin-induced adipogenesis and inflammatory responses. Anti-inflammatory drugs including glucocorticoid, phosphodiesterase inhibitors, and salicylates stimulate insulin-induced adipogenesis of 3T3-L1 cells, and molecular targets for anti-inflammatory drugs such as p38, TNF-α, and IL-1 are involved in adipogenesis or insulin resistance of somatic cells (11, 17–19). Although the molecular mechanism of the cross-talk remains unclear, these lines of evidence implicate the presence of anti-inflammatory compounds in the pool of the adipogenesis-enhancing chemicals. We assayed the 72 adipogenesis-enhancing chemicals for their ability to reduce the production of three inflammatory cytokines, IL-6, IL-2, and TNF-α. Eighteen compounds inhibited the production of a cytokine >50% at 10 μM without notable cytotoxicity, suggesting a high density of cytokine production inhibitors in the adipogenesis-enhancing chemicals. Among those, the compound that we call 69A10 inhibited the TNF-α production in macrophage RAW cells with a IC_{50} of 0.3 μM (Fig. 3). A focused library of adipogenesis-enhancing chemicals may be useful for identifying anti-TNF-α compounds, and their mechanistic studies would clarify the interesting cross-talk between adipogenesis and inflammatory responses.

Osteogenesis Stimulators—Insulin shares sequence homology and biological activity with IGFs. Deficiency in IGF1, a prominent member of IGFs, is suggested to be a cause of decrease in bone density with aging (20, 21), and administration of IGF1 prevents the decrease of bone density in osteoporosis patients in part by stimulating osteogenesis (22–24). The high homology between IGF1 and insulin suggested that the osteogenesis-enhancing activity of IGF1 may be mimicked by the chemicals that potentiated the insulin-induced adipogenesis. As a quick test, the adipogenesis-enhancing chemicals were assayed for their ability to stimulate the formation of bonelike mineral deposition in MC3T3-E1 cells. We found three compounds that increased the mineralization at 5 μM as much as IGF1 or ipriflavone, a clinically used anti-osteoporosis drug (Fig. 4*A*). Their osteogenesis-stimulating activity was confirmed by RT-PCR analysis of osteocalcin, a marker gene of osteoblastic differentiation. The three compounds exhibited an

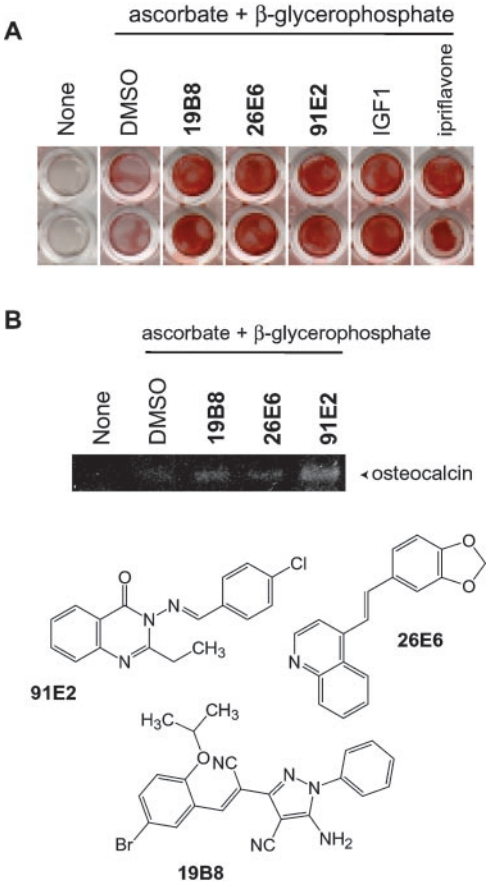

FIG. 4. **Effects of 19B8, 26E6, and 91E2 on the osteogenesis of MC3T3-E1 cells.** *A*, mineralization assay. MC3T3-E1 cells were treated with 1% (v/v) Me_2SO (*DMSO*) or 5 μM 19B8, 26E6, or 91E2 for 14 days, and mineral deposits were stained by Alizarin Red. It is evident that 19B8, 26E6, and 91E2 stimulate the formation of bonelike mineral deposits. Effects of IGF1 (10 ng/ml) and ipriflavone (10 μM) are shown as a positive control. *B*, RT-PCR analysis of osteocalcin. MC3T3-E1 cells were treated with chemicals for 3 days, and total RNA was isolated for RT-PCR analysis.

increased induction of osteocalcin after 3 days of incubation (Fig. 4*B*). These compounds may serve as a small molecule tool for the mechanistic analysis of osteogenesis, and such studies could lead to the development of pharmaceuticals for osteoporosis, one of the most underdiagnosed and undertreated disorders in medicine.

Suppressors of IGF-activated Cancer Cells—We next turned our attention to the 87 compounds that blocked the insulin-induced adipogenesis. Both insulin and IGFs stimulate oncogenic signaling pathways including those of Ras-MAPK and phosphatidylinositol 3-kinase-Akt, and overexpression of IGFs is often associated with cancer malignancy (25). Patients with IGF-overexpressing tumors tend to have severe hypoglycemia despite low levels of serum insulin (known as non-islet cell tumor hypoglycemia) (26), demonstrating a functional overlap between oncogenic IGFs and insulin *in vivo*. These considerations led to the hypothesis that the pool of the adipogenesis-blocking chemicals contains anti-cancer compounds that suppress the IGF-stimulated survival and proliferation of malignant tumor cells. We first examined whether the adipogenesis-blocking chemicals impair the viability of human hepatocellular carcinoma cells that overexpress IGF2, a member of IGFs that is often produced at high levels in liver tumors (27). We identified three chemically analogous compounds that killed IGF2-overexpressing hepatocellular carcinoma cells (Hep-G2) but had milder effects on the cell line with low levels

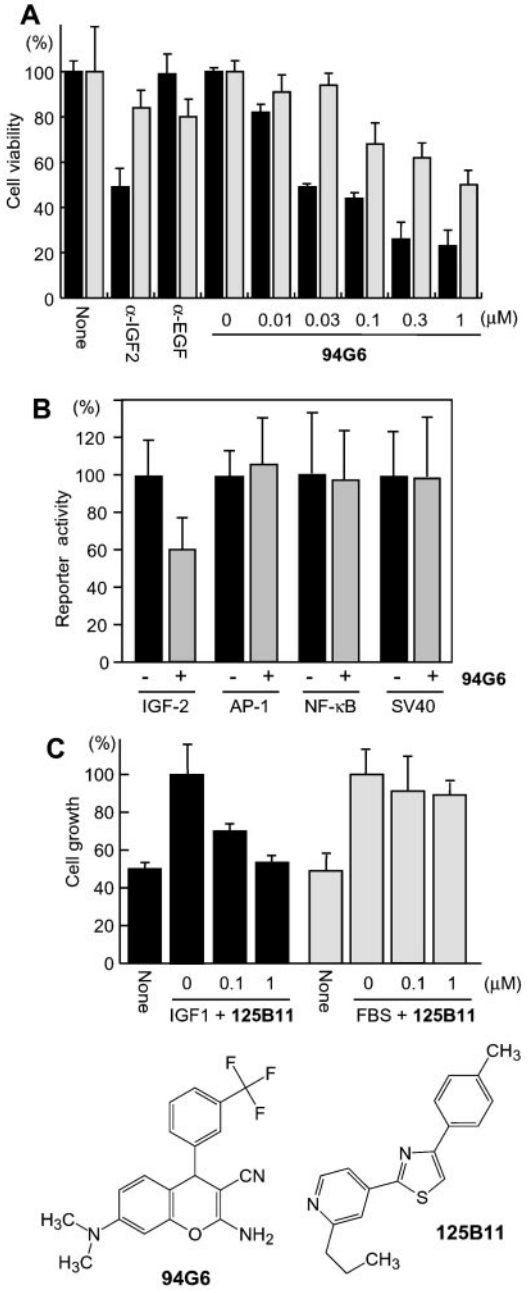

FIG. 5. **Discovery of anti-cancer compounds from the adipogenesis-blocking chemicals.** *A*, hepatocellular carcinoma cell lines, Hep-G2 (*black bars*) and SK-Hep-1 (*gray bars*), were treated with varied amounts of 94G6. 94G6 selectively impaired the viability of IGF2-overexpressing Hep-G2 but had much milder effects on SK-Hep-1 with low levels of IGF2. 94G6 was as selective as a neutralizing antibody against IGF2 (100 μg/ml). The cell viability was estimated by MTT assays in triplicate. *B*, specific inhibition of the IGF2 promoter by 94G6. Hepatocellular carcinoma cells were transiently transfected with a reporter construct in which a gene encoding SEAP is controlled by the IGF2 promoter, AP-1 sites, κB sites, or the SV40 promoter. The transfected cells were treated with 0.1 μM 94G6 for 8 h, and SEAP activity was measured through fluorescence change of a fluorogenic substrate. *C*, 125B11 inhibited the IGF1-induced growth but not the serum-induced growth. DU-145 cells were treated with varied amounts of 125B11 in the presence of IGF1 or 2% fetal bovine serum (*FBS*).

of IGF2 (SK-Hep-1) (28). Repeated experiments with three additional human hepatocellular carcinoma cell lines that we recently characterized[2] indicated that one of the three chemicals, 94G6, exhibited the highest cytotoxicity to IGF2-producing hepatocellular carcinoma cells with selectivity similar to that of a neutralizing antibody against IGF2 (Fig. 5*A*). This

benzochromene derivative killed the IGF2-producing cells at an IC_{50} of 29 nM but had ~33 times weaker effects on the hepatocellular carcinoma cells with low level of IGF2. Reporter gene transcription assays showed that 94G6 selectively inhibits the promoter of IGF2 in the hepatocellular carcinoma cells, suggesting that 94G6 blocks the autocrine loop of IGF2 (Fig. 5*B*). Although 94G6 may target multiple cellular events for causing cell death, the selective inhibition of the IGF2 autocrine loop provides a reasonable explanation for its inhibitory effects on adipogenesis and cancer cell survival.

Another type of IGF-associated tumors is prostate cancer, one of the most common malignant tumors in Western countries. Elevated levels of circulating IGF1 are strongly associated with the risk of developing prostate cancer, and modulation of IGF1 functions by small molecules is an attractive therapeutic approach when combined with androgen-targeting therapies (29). For a chemical screen, we used DU-145 androgen-independent prostate cancer cells whose growth can be stimulated by IGF1 by as much as 2% serum. The pool of the adipogenesis-blocking chemicals contained two analogous chemicals that specifically inhibited the IGF1-induced growth of DU-145 cells but had little effects on their serum-induced growth. One of them, 125B11, had the greatest differential activity in which the simple druglike thiazole derivative impaired the IGF1-induced growth at an IC_{50} of 0.1 μM but had little effects on the serum-dependent growth (Fig. 5*C*). IGF1-induced phosphorylation of Akt and MAPK in DU-145 cells was unaffected by 125B11, suggesting that 125B11 inhibits the cell-proliferative function of IGF1 in a way independent of the known IGF1-signaling pathway. Deregulation of the IGF axis is associated with the initiation and progression of many types of human carcinoma including breast (30) and colorectal cancers (31). A focused library of adipogenesis-blocking chemicals may serve as a source of anti-proliferative agents against the IGF-linked cancers.

DISCUSSION

Fat cell differentiation *per se* has no direct link to glucose uptake, cytokine inhibition, osteogenesis, and selective suppression of cancer cells. Nevertheless, our proof-of-principle study using a 10,000-compound library successfully identified non-cytotoxic bioactive compounds for these seemingly disparate pharmacological effects, just as genetics has identified nonlethal disease-linked genes by examining the eye morphology of fruit flies. We randomly picked up 70 compounds that had no detectable phenotypes in the adipogenesis profiling and assayed for their ability to modulate glucose uptake, cytokine production, IGF-selective cytotoxicity, and osteogenesis. As expected, no significant hits were found in each assay, indicating that the adipogenesis profiling with 3T3-L1 cells is a good filter at least for these pharmacological effects. A data base search revealed that one of the adipogenesis-enhancing chemicals has been patented as an inhibitor of neuropeptide Y, a proposed attenuator of insulin and leptin that stimulates appetite (32). Neuropeptide Y inhibitors are expected to treat feeding disorders and heart diseases (33). Adipogenesis profiling may find use in discovering chemicals with such biological effects. The insulin family of hormones is involved in many other conditions as observed in the complications of hyperinsulinism. The insulin-linked pharmacological effects including wound healing and anti-apoptosis (34) may be expected in adipogenesis-modulating compounds.

One potential drawback of our approach is that the bioactive molecules from the adipogenesis-based focused library may have side effects that are associated with adipogenesis. However, some degree of side effects are usually expected for any unoptimized molecules, and classical medicinal chemistry approaches have been taken for reducing the unwanted side effects. The high sensitivity of the morphological transformation of 3T3-L1 cells also suggests that the adipogenesis-modulating effects of chemicals may not necessarily be reproduced in human. For instance, non-steroidal anti-inflammatory drugs and phosphodiesterase inhibitors such as aspirin and caffeine are known to enhance adipogenesis of 3T3-L1 cells but have no significant effects on fat accumulation in human. The adipogenesis profiling is perhaps a good filter for lead-like bioactive molecules that can be used for further biological, chemical genetic, and medicinal chemical studies.

Adipogenesis-based profiling of more chemical compounds including clinically proven drugs would catalog the biological activities of small organic molecules and help to design a focused chemical library that is small enough to be screened with unique low throughput assays yet generates drug seeds for a broad range of disease conditions. Systematic chemical genetic studies on morphological changes of cells could provide small molecule tools for biological studies of human diseases as found in the role of developmental biology in the analysis of disease-linked genes.

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REFERENCES

1. Thomas, B. J., and Wassarman, D. A. (1999) *Trends Genet.* **15,** 184–190
2. Wassarman, D. A., Therrien, M., and Rubin, G. M. (1995) *Curr. Opin. Genet. Dev.* **5,** 44–50
3. Luo, H., and Dearolf, C. R. (2001) *Bioessays* **23,** 1138–1147
4. McCall, K., and Steller, H. (1997) *Trends Genet.* **13,** 222–226
5. Burke, R., and Basler, K. (1997) *Curr. Opin. Neurobiol.* **7,** 55–61
6. Min, K. T., and Benzer, S. (1999) *Science* **284,** 1985–1988
7. Rosen, E. D., and Spiegelman, B. M. (2000) *Annu. Rev. Cell Dev. Biol.* **16,** 145–171
8. Klemm, D. J., Leitner, J. W., Watson, P., Nesterova, A., Reusch, J. E., Goalstone, M. L., and Draznin, B. (2001) *J. Biol. Chem.* **276,** 28430–28435
9. Ho, I. C., Kim, J. H., Rooney, J. W., Spiegelman, B. M., and Glimcher, L. H. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 15537–15541
10. Dowell, P., Flexner, C., Kwiterovich, P. O., and Lane, M. D. (2000) *J. Biol. Chem.* **275,** 41325–41332
11. Engelman, J. A., Lisanti, M. P., and Scherer, P. E. (1998) *J. Biol. Chem.* **273,** 32111–32120
12. Engelman, J. A., Berg, A. H., Lewis, R. Y., Lin, A., Lisanti, M. P., and Scherer, P. E. (1999) *J. Biol. Chem.* **274,** 35630–35638
13. Iwamura, M., Sluss, P. M., Casamento, J. B., and Cockett, A. T. (1993) *Prostate* **22,** 243–252
14. Komarov, P. G., Komarova, E. A., Kondratov, R. V., Christov-Tselkov, K., Coon, J. S., Chernov, M. V., and Gudkov, A. V. (1999) *Science* **285,** 1733–1737
15. Mayer, T. U., Kapoor, T. M., Haggarty, S. J., King, R. W., Schreiber, S. L., and Mitchison, T. J. (1999) *Science* **286,** 971–974
16. Lehmann, J. M., Moore, L. B., Smith-Oliver, T. A., Wilkison, W. O., Willson, T. M., and Kliewer, S. A. (1995) *J. Biol. Chem.* **270,** 12953–12956
17. Ohsumi, J., Sakakibara, S., Yamaguchi, J., Miyadai, K., Yoshioka, S., Fujiwara, T., Horikoshi, H., and Serizawa, N. (1994) *Endocrinology* **135,** 2279–2282
18. Petruschke, T., and Hauner, H. (1993) *J. Clin. Endocrinol. Metab.* **76,** 742–747
19. Zick, Y. (2001) *Trends Cell Biol.* **11,** 437–441
20. Rosen, C. J., and Donahue, L. R. (1998) *Proc. Soc. Exp. Biol. Med.* **219,** 1–7
21. Baker, J., Liu, J. P., Robertson, E. J., and Efstratiadis, A. (1993) *Cell* **75,** 73–82
22. Bianda, T., Hussain, M. A., Glatz, Y., Bouillon, R., Froesch, E. R., and Schmid, C. (1997) *J. Intern. Med.* **241,** 143–150
23. Ebeling, P. R., Jones, J. D., O'Fallon, W. M., Janes, C. H., and Riggs, B. L. (1993) *J. Clin. Endocrinol. Metab.* **77,** 1384–1387
24. Grinspoon, S., Baum, H., Lee, K., Anderson, E., Herzog, D., and Klibanski, A. (1996) *J. Clin. Endocrinol. Metab.* **81,** 3864–3870
25. Yu, H., and Rohan, T. (2000) *J. Natl. Cancer. Inst.* **92,** 1472–1489
26. Daughaday, W. H. (1995) *Diabetes Rev.* **3,** 62–72
27. Scharf, J. G., Dombrowski, F., and Ramadori, G. (2001) *Mol. Pathol.* **54,** 138–144
28. Zvibel, I., Halay, E., and Reid, L. M. (1991) *Mol. Cell. Biol.* **11,** 108–116
29. Djavan, B., Waldert, M., Seitz, C., and Marberger, M. (2001) *World J. Urol.* **19,** 225–233
30. Sachdev, D., and Yee, D. (2001) *Endocr. Relat. Cancer* **8,** 197–209
31. Hassan, A. B., and Macaulay, V. M. (2002) *Ann. Oncol.* **13,** 349–356
32. Deleted in proof
33. Balasubramaniam, A. (2002) *Am. J. Surg.* **183,** 430–434
34. Dore, S., Kar, S., and Quirion, R. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 4772–4777